Expression of IL-10 and IL-10 Receptors on Peripheral Blood Lymphocytes and Monocytes in Human Head and Neck Squamous Cell Carcinoma

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Objective: Interleukin-10 (IL-10) can mediate anti-tumor activity or tumor escape from the immune system. Here, we investigate IL-10 and IL-10recepter (IL-10R) expression in/on immune cells of patients with head and neck squamous cell carcinoma (HNSCC) and healthy volunteers as normal controls (NC) to further evaluate the dual role IL-10 might play in this disease.
Methods: Peripheral blood mononuclear cells from 15 HNSCC patients and 15 NC were stained and used for flow cytometry or stimulated first with OKT3/anti-CD28 Abs in the presence of IL-2 or with lipopolysaccharide and then stained for flow cytometry. The percentages of IL-10$^+$ or IL-10R$^+$ lymphocytes and monocytes and their mean fluorescence intensity were determined.
Results: Monocytes had the highest frequency of IL-10$^+$ and IL-10R$^+$ cells before or after stimulation and the highest expression levels of these markers followed by CD8$^+$ and then CD4$^+$ T lymphocytes. No significant differences in the frequency or expression levels of IL-10 or IL-10R were observed between patients with HNSCC and NC.
Conclusions: Monocytes in the circulation of patients and NC are the main subset of IL-10- and IL-10R-expressing cells. The frequency of IL-10$^+$ and IL-10R$^+$ monocytes in patients with HNSCC is comparable to that in NC.

Key words: IL-10, IL-10R, monocytes, lymphocytes, head and neck squamous cell carcinoma

INTRODUCTION

In early disease, patients with head and neck squamous cell carcinomas (HNSCC) have benefited from recent progress in surgical techniques, chemotherapy and radiation therapy. However, survival rates in advanced disease, including metastasis to lymph nodes, have not improved in many years [1, 2]. To solve this problem, it is necessary to understand the molecular mechanisms of tumor progression and its interactions with the host immune system. Cytokines play a critical role in tumor progression. In cancer, many cytokines are produced either by the host immune cells or tumor cells or both and can either stimulate or inhibit tumor growth. Therefore, their biology and role in disease are complex.

So far more than 150 cytokines have been identified and classified into two major categories: T helper 1 (Th1) and T helper 2 (Th2) type cytokines. Th1-type cytokines drive cellular immunity to fight intracellular pathogens, including viruses, and to remove cancerous cells. Th2-type cytokines control humoral immunity by up-regulating antibody production to protect against extracellular pathogens. The Th1/Th2 balance reflects the ability of the immune system to eliminate infectious agents and tumors [3, 4]. In general, immune responses in cancer patients have been shown to be biased toward the secretion of Th2-type cytokines, which prevents the generation of effective antitumor Th1 immune response. In fact, decreased levels of Th1-type cytokines have been observed in patients with HNSCC [5].

Interleukin-10 (IL-10) is a pleiotropic cytokine with the ability to modulate immunoregulation and inflammation in the context of adaptive and innate immunity. Although IL-10 is classified as a Th2-type cytokine, it is now known to be produced by many different cell types. Recent evidence shows that Th1 cells are the main source of IL-10 which controls immune responses against some bacterial infections. Moreover, IL-10 is involved in the feedback control of Th1 cells [6]. However, the role of IL-10 in immunoregulation is controversial. Although various cells mediating suppression, including tumor cells, produce IL-10, monocytes and regulatory T cells (Treg) are its main source [7–11]. IL-10 binds to the Interleukin-10 receptor (IL-10R) situated on the cell surface, which consists of α and β subunits [12, 13] and belongs to class II cytokine receptors broadly expressed on thymocytes, T cells, B cells, NK cells, monocytes and macrophages. These receptors signal via the Jak/Stat pathway. Ligand binding to IL-10R induces Jak1 and Tyk, resulting in STAT1 and STAT3 activation. Upon dimerization of STATs and their translocation to the nucleus, nu-

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Table I Clinicopathologic characteristics of patients with HNSCC who donated blood for this study.

Age Range in yrs. 35–81	
Sex	
Male	10
Female	5
Tumor Site	
Oral Cavity	10
Oropharynx	4
Hypopharynx	1
Tumor Differentiation	
Poor	1
Moderate	9
Well	1
Not Determined	4
Tumor Stage	
T_1	2
T_2	5
T_3	3
T_4	3
Unstaged	2
Nodal Status	
N_0	11
N_1	2
N_2	0
N_3	0
Unstaged	2

merous target genes are transactivated [14–16]. IL-10 knockout mice exhibit severe intestinal inflammation in response to normal flora [17]. Therefore, IL-10 is commonly regarded as an anti-inflammatory or immunosuppressive cytokine, and this suggests that IL-10 plays an important role in immune tolerance. It has been generally considered that IL-10 is an immunosuppressive cytokine secreted by tumors (or tumor-infiltrating immune cells) which allows malignant cells to escape from immune surveillance [18–20]. In contrast, other preclinical and clinical data suggest that IL-10 might favor immune-mediated rejection of cancer [21–24]. Thus, IL-10 might have several distinct roles in mediating cancer progression. IL-10 inhibits macrophage and dendritic cell (DC) activity. Its suppressive functions consequently lead to the inhibition of pro-inflammatory (Th1) cytokine secretion and of the MHC class II and co-stimulatory molecule expression. In turn, this results in suppression of the antigen processing machinery and of tumor-associated antigen presentation by antigen-presenting cell (APC) [25–29]. IL-10 also down-regulates TAP1 and TAP2 expression, giving rise to accumulation of MHC I molecules in the endoplasmic reticulum. It protects tumor cells from cytotoxic T-lymphocyte (CTL)-mediated cytotoxicity [30]. IL-10 also has several roles as a mediator of anti-tumor activity. It enhances NK cell activity thus promoting killing of tumor cells. It might enhance release of antigens from damaged cells, and it inhibits maturation of APCs thus increasing their ability to uptake antigens and to remain *in situ* rather than migrate to regional lymph nodes [31, 32]. IL-10 also suppresses tumor angiogenesis and invasiveness through induction of metalloproteinase (MMP) inhibitors [33–35]. Moreover, IL-10, through anti-inflammatory effects, can lead to suppression of tumor growth since chronic inflammation is highly associated with tumor initiation and progression [30, 36]. IL-10 also inhibits NF-κB activation and thus blocks production of proinflammatory cytokines. It might lead to tumor regression though anti-inflammatory effects [36–38].

As indicated above, IL-10 can either promote anti-tumor activity or mediate tumor escape from immune surveillance. However, the dual role of IL-10 in health and disease remains unclear. In this study, we investigated IL-10 and IL-10R expression in/on $CD4^+$ or $CD8^+$ lymphocytes and monocytes in the peripheral blood of HNSCC patients and healthy volunteers to further evaluate these dramatically opposing roles of IL-10 in cancer.

MATERIALS AND METHODS

Head and neck squamous cell carcinoma (HNSCC) patients and healthy volunteers

Blood samples were obtained from 15 HNSCC patients and 15 healthy volunteers as normal controls (NC). All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh. All patients were seen at the Outpatient Otolaryngology Clinic at the University of Pittsburgh Cancer Institute (UPCI) between August 2008 and December 2008. The patient cohort included 9 males and 6 females. The NC group included 10 males and 5 females. All 15 patients had untreated primary tumors. The age, sex, and clinicopathologic characteristics of the patients are listed in Table I.

Collection of peripheral blood mononuclear cells

Peripheral venous blood (20–50 mL) was drawn into heparinized tubes. The samples were hand-carried to the laboratory and immediately centrifuged on Ficoll-Hypaque. Peripheral blood mononuclear cells (PBMC) were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments.

Cell culture

PBMC for measuring IL-10 or IL-10R in lymphocytes were cultured in a complete medium consisting of AIM V supplemented with 10% (v/v) ΔFCS, 100 IU/mL penicillin, 100 μg/mL streptomycin and L-glutamine (2 mmol/l) at 37°C in an atmosphere of 5% CO_2 in air. PBMC were divided into two groups: (a) PBMC (1 x 10^6 cells/mL) were stimulated with OKT3 (1 μg/mL), anti-CD28 (1 μg/mL) and 100 IU IL-2/mL for 24 h; (b) PBMC were not stimulated. PBMC for measuring IL-10 or IL-10R in monocytes were similarly cultured in a complete medium consisting of RPMI 1640 supplemented with 10% (v/v) ΔFCS, 100 IU/mL penicillin, 100 μg/mL streptomycin and L-glutamine (2 mmol/l) at 37°C in an atmosphere of 5% CO_2 in air. PBMC were divided into two groups: one stimulat-

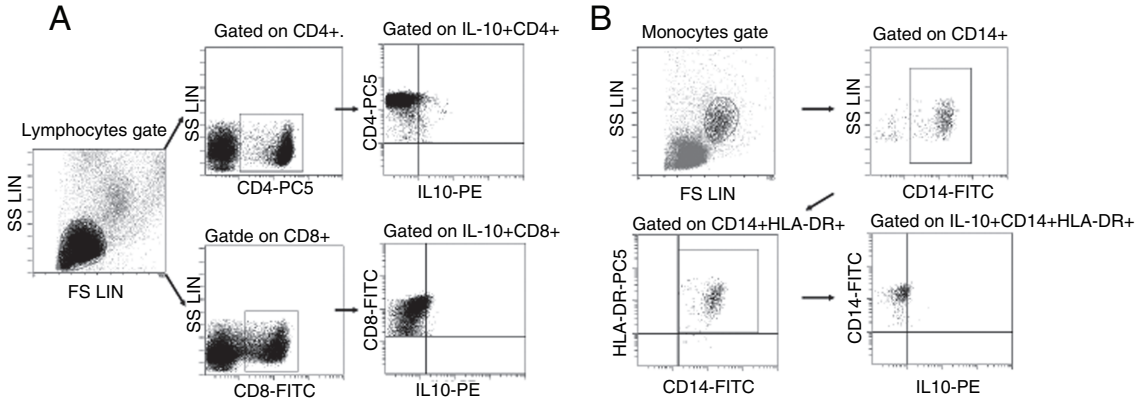

Fig. 1 Gating strategy for analysis of IL-10 or IL-10R expression in PBMC lymphocyte and monocyte subsets. The acquisition and analysis gates were restricted to the lymphocyte (A) or monocyte (B) gates as determined by the characteristic forward scatter (FS) and side scatter (SS) properties of these cells. For further analysis, gates were restricted to the $CD4^+$ or $CD8^+$ T cells (A) or $CD14^+HLA\text{-}DR^+$ monocyte subsets (B). The frequency of $IL\text{-}10^+$ or $IL\text{-}10R^+$ cells was measured in each subset.

ed with lipopolysaccharide (LPS) (5 μg/mL) for 12 h before harvest and the other not stimulated. Monensin (1 μg/mL; GolgiStop; BD Pharmingen) was added for 4 h before harvesting cells for flow cytometry.

Antibodies

The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD8-FITC, anti-CD4-PC5, anti-CD14-FITC, anti-HLA-DR-PC5, anti-IL-10-PE, anti-CD210 (IL-10 R)-PE. Antibodies and their respective isotypes (negative controls) used for surface or intracellular staining, were all purchased from Beckman Coulter (Miami, FL), except for anti-IL-10-PE and its isotype control PE rat $IgG_{2a,\kappa}$ (BD Pharmingen, San Jose, CA), anti-CD210 (IL-10 R) PE and its isotype control PE Rat $IgG2_{a,\kappa}$ (Biolegend, San Diego, CA). Prior to use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions.

Surface and intracellular staining

PBMC samples were first incubated with mAbs specific for CD8 and CD4 markers expressed on lymphocytes or CD14 and HLA-DR expressed on monocytes for 30 min at room temperature (RT). Anti-IL-10R Ab was added to each sample. After extensive washing, cells were fixed with 2% (w/v) paraformaldehyde in PBS for 20 min and prepared for flow cytometry. Appropriate isotype controls were used in all experiments. To stain for intracytoplasmic IL-10, cells were washed once with PBS containing 0.5% (w/v) BSA and 2 nM EDTA and permeabilized with PBS containing 0.5% BSA and 0.2% (w/v) saponin for 20 min. Cells were stained with anti-IL-10-PE Ab or its isotype control. Cells were further washed twice with PBS containing 0.5% BSA and 0.2% saponin, resuspended in FACS flow solution and immediately analyzed by flow cytometry.

Flow cytometry

Flow cytometry was performed using a FACScan flow cytometer (Beckman Coulter) equipped with Expo32 software (Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte or monocyte gates as determined by the characteristic forward scatter (FS) and side scatter (SS) properties of these cells. FS and SS coordinates were set in a linear scale. The analysis gates were restricted to the $CD4^+$ and $CD8^+$ T cell subsets or $CD14^+HLA\text{-}DR^+$ monocyte subsets. Fig. 1 shows the gating strategy for analysis of IL-10 or IL-10R expression on each cell subset.

Statistical analysis

The statistical difference was determined by two-sided Student's test. Differences with $p < 0.05$ were considered to be significant.

RESULTS

The peak time for IL-10 expression in lymphocytes and monocytes.

The peak time of IL-10 expression after stimulation depends on the cell type. To determine the peak time for IL-10 expression in lymphocytes and monocytes in human PBMC, we performed flow cytometry at various time points after stimulation of lymphocytes with OKT3 (1 ug/mL), anti-CD28 (1 ug/mL), IL-2 (100 IU/mL), and of monocytes with LPS (5 ug/mL). The peak time of IL-10 expression was 24 h for lymphocytes and 12 h for monocytes after cell stimulation (data not shown).

Frequency of lymphocyte and monocytes in PBMC obtained from HNSCC patients and healthy donors.

To evaluate the frequency of lymphocytes and monocytes in PBMC of HNSCC patients and NC, we performed flow cytometry. The percentages of lymphocytes and monocytes in PBMC of HNSCC patients and NC were comparable (Table II).

IL-10 expression in lymphocytes and monocytes in HNSCC patients and healthy donors.

To investigate IL-10 expression in lymphocytes and monocytes in PBMC of HNSCC patients and NC, we measured the percentages of positive cells and their

Table II Frequency of lymphocytes and monocytes in PBMC of HNSCC patients and normal donors.[a]

	Normal Donors (n = 15)	Patients (n = 15)	P-value
Lymphocytes	83 ± 9	77 ± 10	NSD
$CD4^+$ cells	68 ± 9	63 ± 13	NSD
$CD8^+$ cells	16 ± 7	22 ± 10	NSD
Monocytes	5 ± 2	5 ± 2	NSD
$CD14^+HLA\text{-}DR^+$ cells	87 ± 8	88 ± 5	NSD

[a] The values are mean % ± SD

NSD = no significant difference and between NC and patients

Table III Percentages and MFI of $IL\text{-}10^+$ and $IL\text{-}10R^+$ lymphocytes and monocytes tested prior to and after stimulation. [a]

	IL-10 expression (%)		MFI	
	No Stimulation	Stimulation	No Stimulation	Stimulation
NC				
$CD4^+$	1 ± 1	3 ± 2	1 ± 0	1 ± 1
$CD8^+$	3 ± 2	3 ± 3	1 ± 0	1 ± 0
$CD14^+HLA\text{-}R^+$	12 ± 8	21 ± 7	1 ± 1	2 ± 2
HNSCC				
$CD4^+$	2 ± 2	3 ± 2	1 ± 0	1 ± 0
$CD8^+$	4 ± 4	6 ± 6	1 ± 0	1 ± 0
$CD14^+HLA\text{-}R^+$	10 ± 6	20 ± 8	1 ± 0	1 ± 1
	IL-10R expression (%)		MFI	
	No Stimulation	Stimulation	No Stimulation	Stimulation
NC				
$CD4^+$	4 ± 3	2 ± 3	1 ± 0	1 ± 1
$CD8^+$	7 ± 5	3 ± 2	1 ± 0	1 ± 0
$CD14^+HLA\text{-}R^+$	18 ± 7	11 ± 7	1 ± 1	2 ± 1
HNSCC				
$CD4^+$	3 ± 1	2 ± 1	1 ± 0	1 ± 0
$CD8^+$	9 ± 7	4 ± 4	1 ± 0	1 ± 0
$CD14^+HLA\text{-}R^+$	16 ± 8	11 ± 8	1 ± 0	2 ± 2

[a] Cells were obtained from patients with HNSCC and normal donors as normal controls (NC). Following stimulation with OKT3/anti-CD28 Abs in the presence of IL-2 for lymphocytes or with LPS for monocytes, IL-10 and IL-10R expression in each cell subset were determined by flow cytometry. The values are mean % ± SD

MFI prior to and after stimulation of PBMC with OKT3 and anti-CD28 Ab+ IL-2 for lymphocytes or with LPS for monocytes. Fig. 2A shows that unstimulated $CD4^+$ and $CD8^+$ T lymphocytes expressed little IL-10, but upon stimulation up-regulated its expression. In contrast, monocytes expressed IL-10 before stimulation and up-regulated its expression further after LPS treatment. Fig. 3A shows percentages of positive cells for IL-10 before and after stimulation in PBMC of NC and patients with HNSCC. After stimulation, the mean percentage of $IL\text{-}10^+$ cells in NC significantly increased in $CD4^+$ lymphocytes and $CD14^+HLA\text{-}DR^+$ monocytes but not in $CD8^+$ T lymphocytes (Fig. 3A). In HNSCC patients, only $IL\text{-}10^+CD14^+HLA\text{-}DR^+$ monocytes significantly increased in the frequency after stimulation (Fig. 3A). There was no statistically significant difference between HNSCC patients and NC in the percentages of $IL\text{-}10^+$ cells before or after stimulation. However, higher percentages of $CD14^+HLA\text{-}DR^+$ monocytes than lymphocytes were positive for IL-10 before or after stimulation in both NC and patients with cancer.

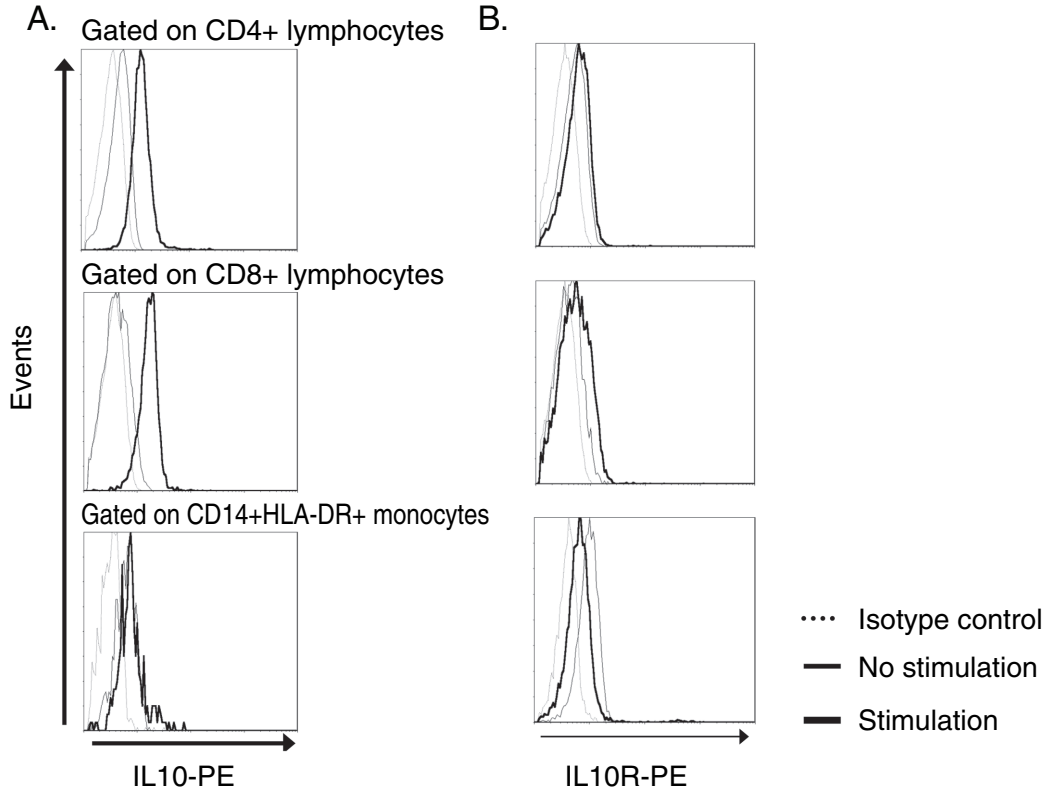

Fig. 2 Representative flow cytometry histograms for analysis of IL-10 (A) or IL-10R (B) expression in unstimulated or stimulated lymphocytes and monocytes.

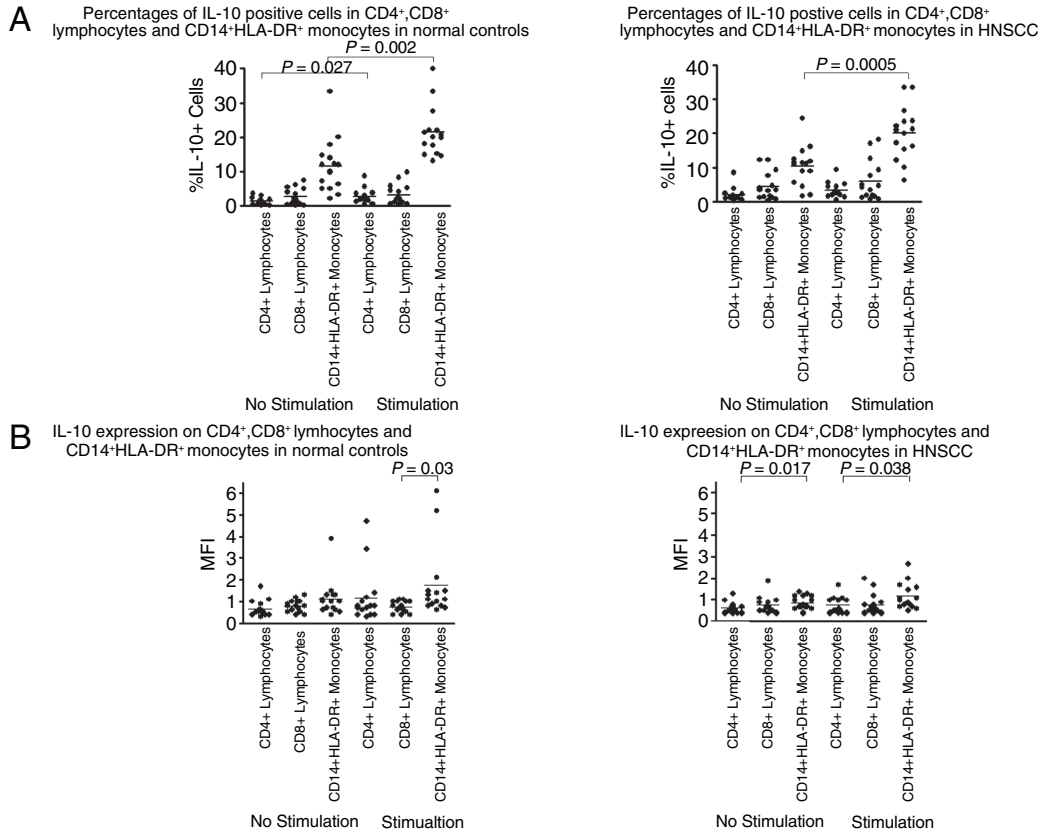

Fig. 3 Percentages (A) and MFI (B) of IL-10$^+$ cells in stimulated or unstimulated lymphocytes and monocytes obtained from normal donors (n = 15) and HNSCC patients (n = 15). Horizontal lines represent median values.

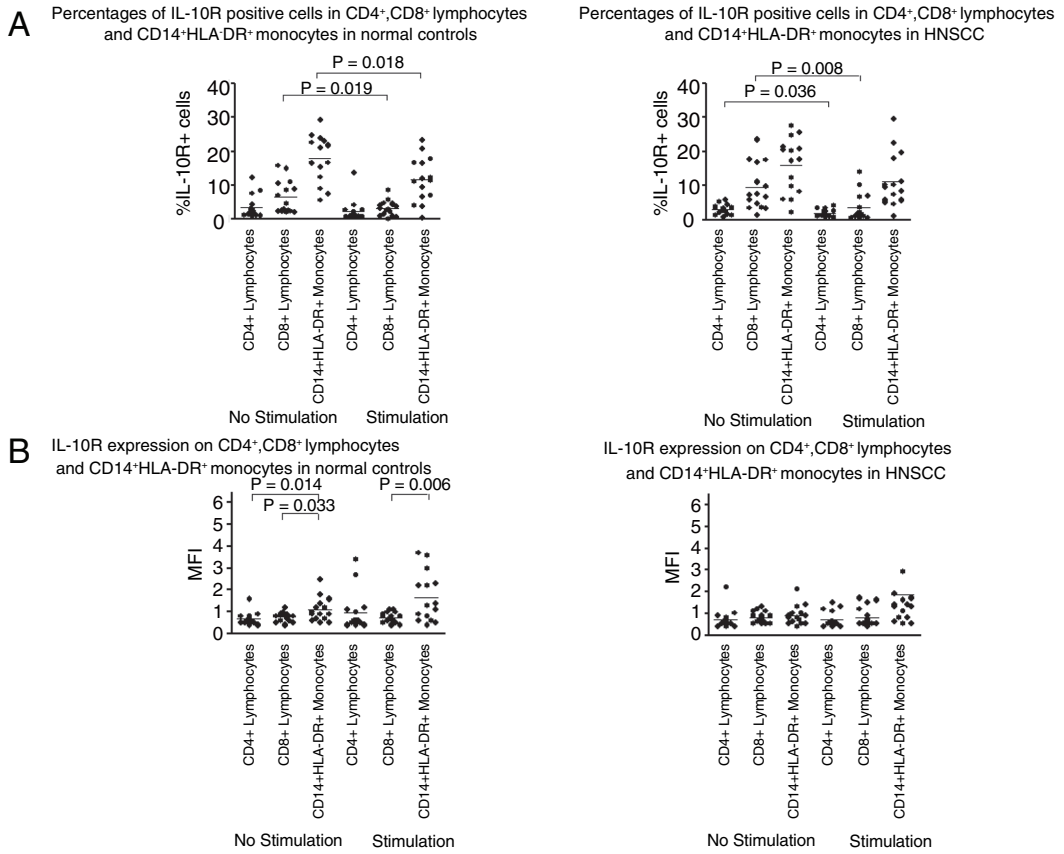

Fig. 4 Percentages (A) and MFI (B) of IL-10R$^+$ cells in stimulated or unstimulated lymphocytes and monocytes obtained from normal donors (n = 15) and HNSCC patients (n = 15). Horizontal lines represent median values.

Expression levels (MFI) of IL-10 were found to be unaffected by stimulation of lymphocytes in NC. However, significant upregulation of IL-10 expression upon LPS stimulation was observed in monocytes obtained from NC (Fig. 3B). In HNSCC patients, unstimulated monocytes had a higher IL-10 expression level than lymphocytes, and upon LPS stimulation, MFI of CD4$^+$, CD8$^+$ and monocytes were elevated relative to unstimulated cells. However, there were no significant differences between HNSCC patients and NC in MFI for IL-10 on lymphocytes or monocytes.

IL-10R expression on lymphocytes and monocytes in HNSCC patients and healthy donors.

To investigate IL-10R expression on lymphocyte subsets and monocytes in PBMC obtained from HNSCC patients and NC, we measured both percentages and MFI by flow cytometry.

As shown in Fig. 2B, a proportion of unstimulated lymphocytes expressed IL-10R and this proportion was increased upon stimulation. In contrast, monocytes down-regulated IL-10R expression, so that fewer cells were IL-10R$^+$ upon LPS stimulation. The percentages of IL-10R$^+$ lymphocytes and monocytes were lower after stimulation in NC as well as in HNSCC patients (Fig. 4A). There were no statistically significant differences between HNSCC patients and NC in the percentage of IL-10R$^+$ lymphocytes or monocytes. The mean MFI for IL-10R was the highest in monocytes before and after stimulation, largely because of the substantial MFI increases in monocytes of several normal donors. In contrast, monocytes in HNSCC patients had uniformly low expression of IL-10R. No significant difference between patients and NC were evident in MFI for IL-10R in either lymphocytes or monocytes (Fig. 4B).

DISCUSSION

Cytokines play a critical role in cancer modulating functions of both tumor and host cells. Therefore, a better understanding of the pathways regulating expression of cytokines is essential to search for novel immunomodulatory interventions in patients with cancer. Each cytokine has its own optimal secretion time from producer cells, depending on the cell type and the method of stimulation. However, cytokine expression does not always result in secretion, especially in pathologic states, and the advantages of measuring cytokine expression is that the cell of its origin can be simultaneously identified by flow cytometry. We observed that the peak time for IL-10 expression in lymphocytes was 24 h after stimulation with OKT3, anti-CD28 and IL-2, while monocytes had the peak time for IL-10 expression of only 12 h after stimulation with LPS (5 ug/mL). Similar data for the time course of IL-10 secretion expression were reported by others [39, 40].

IL-10 plays an important role in immune tolerance,

as it can suppress excessive inflammatory reactions. Therefore, its expression by inflammatory cells, especially following their activation, is of considerable interest in conditions associated with chronic inflammatory states such as those associated with many human cancers. HNSCC are often infiltrated by mononuclear cells, and the frequency of tumor-infiltrating mononuclear cells has been associated with the improved prognosis [41]. There are no data available for IL-10 or IL-10R expression on tumor-infiltrating mononuclear cells in HNSCC. Although this study was performed with the peripheral blood not with tumor specimens, it provides some insights into the potential of immune cells to express IL-10 and signal via the IL-10R.

We observed that in the peripheral blood of NC or HNSCC patients only unstimulated monocytes constitutively expressed IL-10 and IL-10R. Further, no differences in the percentage of IL-10$^+$ monocytes or IL-10 expression levels were evident in patients vs. NC. Few lymphocytes are IL-10$^+$ before or after *ex vivo* stimulation, but the proportion of IL-10$^+$ monocytes and expression levels of IL-10 on monocytes are significantly increased after LPS stimulation. Monocytes also had the highest expression level of IL-10R, and the frequency of IL-10R$^+$ monocytes was around 20% in unstimulated PBMC. It was also interesting to note that a subset of CD8$^+$ T cells (~10%) phenotyped as positive for IL-10R in unstimulated PBMC. This implies that IL-10R$^+$ monocytes and CD8$^+$ T cells are ready to respond to this cytokine even in the absence of exogenous stimuli. Interestingly, the percentages of IL-10R$^+$ lymphocytes and monocytes decreased following stimulation. Thus, stimulated IL-10$^+$ monocytes or lymphocytes down-regulate IL-10R perhaps because of its internalization following complexing with IL-10. In patients with HNSCC this process of IL-10/IL-10R signaling appears to proceed as effectively as in the peripheral blood of NC.

Based on our data, we conclude that monocytes in the circulation of patients with HNSCC and NC are the main subset of IL-10 and IL-10R expressing cells. In the periphery, IL-10 expression appears to be under strict homeostatic control, being restricted to monocytes and LPS-activated monocytes. Thus, in the periphery, IL-10 expression is restricted to cells involved with control of microbial infections, and in NC and in patients with cancer, IL-10$^+$ monocytes play a key role in defense against infectious agents. We also demonstrated that there was no statistically significant difference between HNSCC patients and NC in IL-10 and IL-10R expression in lymphocytes and monocytes in the peripheral circulation. However, in the tumor microenvironment, their frequency and ability to produce IL-10 might be substantially different. Human tumors, including HNSCC, produce IL-10 and tumor-infiltrating lymphocytes, e.g., Treg, are IL-10$^+$ [42]. Tumor-associated macrophages (TAMs) are also producers of IL-10. The dramatically opposing effects of IL-10 might depend on interactions with other cytokines or factors in the tumor microenvironment. Therefore, it will be necessary to further investigate IL-10 expression in healthy tissues as well as in the tumor microenvironment to evaluate its dual role in cancer. In addition, it will be necessary to further explore IL-10 biochemistry, including its secretion and signal transduction to better define the significance of IL-10 in tumor immunology.

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